TARGET DIRECTED DRUG DISCOVERY: A TECHNOLOGY DRIVEN APPROACH TO COMBATING MALARIA

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ABSTRACT

A procedure is described for identifying chemical entities with potential for being developed into antimalarial drugs. State-of-the-art technologies involving molecular genetics, computer modeling, structural analysis and high throughput screening have been leveraged to optimize the chances of success and minimize the time required.

1. INTRODUCTION

Historically, the parasitic disease malaria has been a major concern for U. S. military operations in vast regions of the world. The effectiveness of every antimalarial drug in the field is continually eroded by the development of parasite resistance. With no vaccine available, new drugs must continuously be developed to replace those compromised by emerging resistance. Drugs used to treat malaria disrupt biochemical processes (targets) that are essential to parasite survival. To maximize their useful lifespan, new drugs must hit processes that are distinct from those targeted by the drugs they are replacing. In the past, drug discovery relied solely on empirical screening of large drug libraries for effectiveness in killing parasites *in vitro* - with no concern for target identity. Recently, the Antimalarial Drug Discovery Program at the Walter Reed Army Institute of Research (WRAIR) adopted a target based approach: drug candidates are identified and developed based on their ability to disrupt specific parasite targets. The approach is a modern standard of the pharmaceutical industry. Target based discovery efforts leverage the tremendous advances in molecular biology, computer simulation, structure analysis and high throughput technologies for the identification of lead drug candidates.

Because of the emergence of drug resistant strains of the malaria parasite, new drugs must be fielded continuously as replacements for ineffective predecessors. Ethical, scientific and regulatory concerns prescribe the minimal information needed before a drug can be introduced in man. The plan developed by the Drug Discovery Program (DDP) at WRAIR describes a cost-effective method of selecting from millions of chemical entities those vanishing few having the greatest likelihood of being fielded as effective and safe antimalarials in man. An effective drug has certain physical, chemical and biological characteristics. The DDP has established a series of tests to assess the degree to which a drug meets these characteristics. Temporally, tests are arranged such that inexpensive tests which can eliminate the greatest number of candidate drugs are run first. The test progression advances to increasingly expensive and selective assays. The philosophy of the program is to eliminate a candidate as early as possible in the selection process.

2. BIOLOGY OF THE MALARIA PARASITE

Malaria is caused by a single-celled protozoan of the genus *Plasmodium*. There are four species of *Plasmodium* that may cause malaria in humans: *P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*. *P. falciparum* is the most dangerous, as it can cause severe anemia, kidney failure, and brain damage; it is often fatal, especially among children. In *P. vivax* infection, the typical symptoms - cycles of chills, high fever, and sweating with headache, muscle aches, and nausea – are less severe. Although the disease is not often fatal, relapses can occur periodically for up to 3 years. *P. malariae* infections can persist in the blood without producing symptoms for life; chronic infections in children can lead to kidney damage. Each of the four species has a distinctive appearance and life cycle. The PDP focuses on discovery of drugs to treat both *P. falciparum and P. vivax* infections, the results may apply to other types of malaria infections.

Other species of *Plasmodium* cause malaria in a variety of vertebrates. Several are used as models, including *P. berghei*, *P. yoelli*, *P. vinckei*, and *P. chabaudi* in rates and mice, and *P. knowlesi* in the rhesus monkey. In addition, some human malaria parasites can infect certain higher apes and New World monkeys; the South American owl monkey provides a model for *P. falciparum*.

All malaria parasites have a complex life cycle, alternating between vertebrate hosts and mosquitoes. In vertebrates they reproduce asexually, first in the liver and

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then, repeatedly, in the red blood cells (erythrocytes). In the mosquito, they reproduce sexually.

Human infection begins with the bite of an infected female *Anopheles* mosquito. As a blood meal is ingested, the mosquito simultaneously injects saliva containing plasmodial sporozoites into the human host. The motile, threadlike sporozoites quickly leave the bloodstream and lodge in the cells of the liver as hepatocytes. Within about an hour of the bite, all sporozoites disappear from blood circulation. Over the next week, each sporozoite that has invaded a liver cell becomes a schizont, a developmental structure that contains thousands of merozoites. Liver stage parasites are known as exo-erythrocytic forms, to distinguish them from blood stage erythrocytic forms. When the schizont is mature, it ruptures out of the infected liver cell and discharges thousands of merozoites into the blood stream. In *P. vivax* and *P. ovale* malaria, some sporozoites become hypnozoites, forms that can remain dormant in the liver for months or years before they start to proliferate.

Once merozoites are released into the bloodstream, they invade erythrocytes; *P. vivax* and *P. ovale* parasites invade young erythrocytes, while *P. malariae* preferentially infects mature erythrocytes; *P. falciparum* invades old and young cells alike. This is one reason why the concentration of parasites in the blood reaches dangerously high levels in *P. falciparum*.

Most of the parasites that enter erythrocytes undergo a second round of asexual reproduction, which is similar to but quicker and less prolific than that in the liver cells. In 2 or 3 days, depending on the species, the intra-erythrocytic parasite has developed from young ring forms to trophozoites to the dividing form, again known as a schizont. Red blood cells infected with *P. falciparum* develop small sticky protrusions of parasite origin that allow the infected erythrocytes to adhere to the lining of small blood vessels while the parasite matures.

Depending on the species, each schizont contains 10 to 20 erythrocytic merozoites. When the schizont is mature, these merozoites burst out of the erythrocytes and invade additional red blood cells, thus perpetuating the cycle of infection. It is at this point, when the red blood cells rupture, that clinical symptoms appear. Because the cycle can repeat every 48 hours in *P. vivax*, *P. falciparum*, and *P. ovale*, or every 72 hours in *P. malariae*, attacks of fever can occur every 2 or 3 days.

Plasmodial parasites thus continue to recycle until they are brought under control through drug therapy or through the host's immune defenses, or until the host dies. If reinfection does not occur, *P. falciparum* infections will generally clear in 1 to 2 years; *P. vivax* and *P. malariae* may last 3 years; *P. malariae*, if untreated, can persist as an asymptomatic infection for decades. Some of the merozoites that invade red blood cells, instead of developing asexually, differentiate into sexual forms, called gametocytes. Mature gametocytes, enclosed within the erythrocyte membrane, circulate in the host's blood, available to feeding *Anopheles* mosquitoes.

Blood ingested by the mosquito carries the gametocytes into the mosquito's stomach, where they shed the red blood cell envelope. Male gametocytes rapidly transform into motile, spermlike structures and fertilize the larger, egglike female gametes, forming zygotes. In about a day, oocyte forms develop from the zygotes and then burrow into the mosquito's stomach wall, where they form oocyts. In 9 to 14 days they rupture and release sporozoites that infect the mosquito's salivary gland. One cycle is thus complete.

3. THE DRUG DISCOVERY PLAN

The target-based drug discovery program is based upon the most current tenets of drug discovery used by major pharmaceutical companies. The objective is to identify and validate a critical target in the parasite, that if inhibited will result in the death of the parasite. Current limitations to this approach include an incomplete understanding of the biology of the parasite, a limited ability to manipulate the parasite genome (e.g., knock-outs) to validate targets, and the innate ability of the parasite to become resistant quickly to structurally dissimilar drugs.

The current Drug Discovery program portfolio includes three basic drug discovery approaches: structure- and target-based drug design, natural product drug discovery, and classical medicinal chemistry approaches (Screening of chemical libraries), but with a distinct emphasis on the target-based drug discovery program. All of these approaches are augmented by the iterative use of rational drug design methodologies The manner in which these approaches are assembled and integrated is illustrated in figure 1.

The gold standard for drug discovery is the *in vitro* screen for efficacy against Plasmodium falciparum (fig. 1, yellow box). This assay acts as a selector for compounds which work in the blood stages of the malarial infection. The three approaches for selecting drugs are represented by the three dark blue boxes: compound libraries, natural products and target screens (more on the target screens later). Compounds which demonstrate a minimal level of efficacy (Hits) are subjected to a series of in vitro tests designed to predict

FIGURE 1.

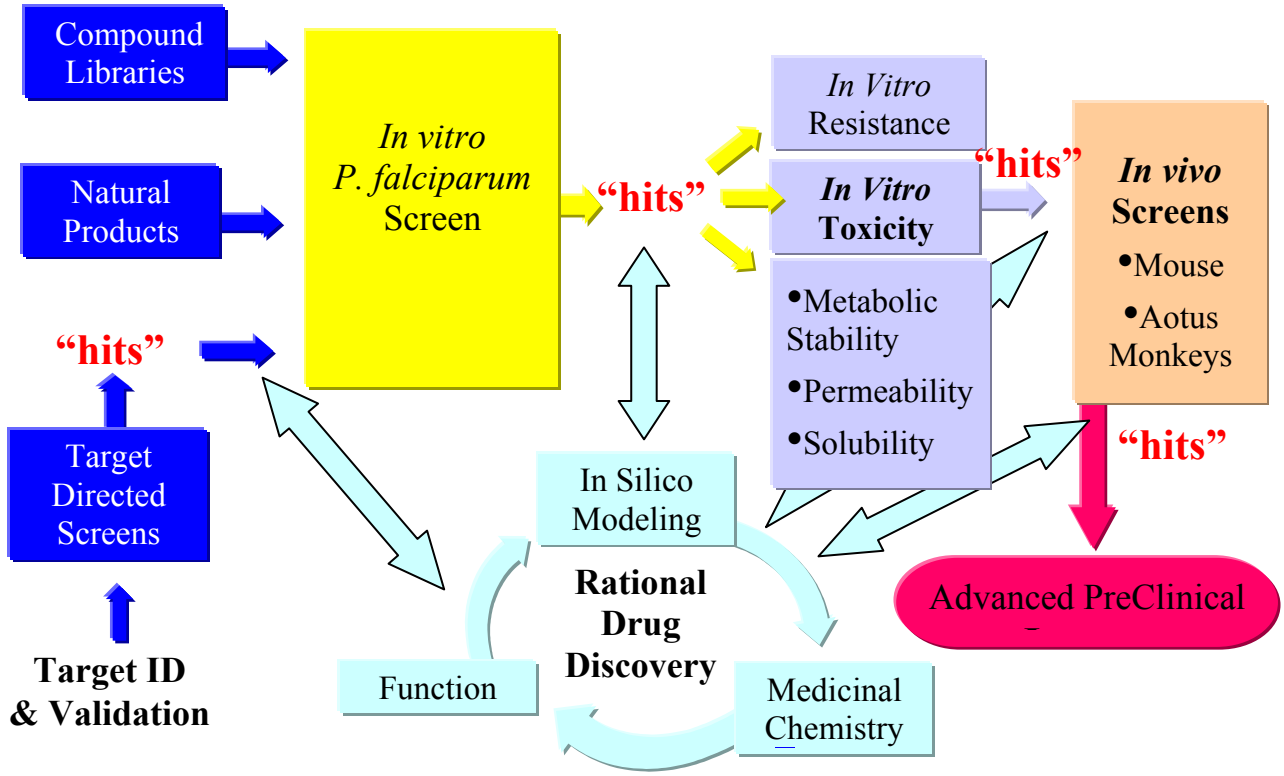

Fig. 1 Overview of the drug discovery plan.

key characteristics (purple boxes): mammalian cell toxicity, resistance, solubility, metabolic stability. Compounds that meet acceptable criteria in these areas would be sent to the first of the *in vivo* efficacy tests: mouse malaria (Thompson Test). This assesses the efficacy of a compound against *P. berghei*. Preliminary toxicity information is also obtained in these tests. Successful candidates are then sent to the Aotus monkey test (brown box), where they are assessed for in vivo activity against the human parasites *P. falciparum* or *P. vivax*. Successful candidates are then sent to advanced preclinical studies (red oval)to include pharmaco-dynamics, pharmacokinetics and toxicity. It is at this stage that a compound will be transitioned to the Drug Development Program.

The process outlined above is linear in nature; multiple steps follow sequentially to arrive at a designated end point. However, another procedure interacts with the drug discovery process at several key points. Rational Drug Discovery or Design (light blue structures, fig 1) is an iterative procedure consisting of : 1. observing the effect of a drug in a specific functional test (for example, the *in vitro P. falciparum* screen or the *in vivo* mouse efficacy test), 2. correlating that effect with the drug's structure and 3. using that structure to select new compounds for assessment in the functional test.

It is important to understand the overall drug discovery process in broad terms. In the next sections, several aspects of the process (target identification and validation, high throughput screening and) which are particularly important to antimalarial drug discovery will be considered in greater detail.

4. THE TARGET DIRECTED APPROACH

4.1 The Target Directed Process

Because of the constant eroding of drug efficacy in the field, new drugs must constantly be deployed. The new drugs should hit targets not used by previously deployed drugs to assure optimal utility and longevity in the field. Target directed drug discovery achieves this goal by selecting only drugs directed against known

targets. Target directed drug discovery is a linear progression of steps:

- Identify and validate parasite target
- Clone *P.falciparum* gene
- Express and purify recombinant protein
- Develop assay to measure activity → HTS
- Establish target structure
- Identify inhibitors
- Develop a pharmacophore
- Define structure-activity relationships
- Produce a lead compound

This process should optimize the probability that only drugs acting on new targets will be developed.

4.2 Impact of Genomics on Drug Discovery

Leveraging the tremendous power of genomics to identify and validate optimal drug candidates is critical to the success of the drug discovery program. The steps of the program that genomics influence include:

- Identification of targets
- Validation of targets
- Acceleration of the Structure-Based Drug Design Program
- Elucidation of resistance mechanisms
- Identification of modes of action for 'empirical' drugs

5. TARGET IDENTIFICATION AND VALIDATION

A drug target is an enzyme, structure or process that is vitally important to the parasite. Targets are identified using information from the literature, bioinformatics and genomics/proteomics (Table 1). What are the criteria that define a "good" target? Table 1 lists a number that are considered in WRAIR's target selection protocol.

Target Discovery

- Literature
- Bioinformatics
- Functional Genomics/Proteomics

Target Selection Criteria

- Essential for Survival
- Functional Assay
- Known Inhibitors (Target)
- Known Inhibitors (Parasite)
- Known Structure
- Human Homologue
- Developmental Stage Profile

Table 1 Target discover and selection criteria.

Ideally, the target would be essential for survival of the parasite. A functional assay for the target should be feasible if not already in existence. Enzymatic assays would be the most straight forward, however, physical changes which are correlated to functional alterations in the target could also be used as endpoints. Known inhibitors of the parasite target (and to a lesser degree, the human target analogue) function accelerates the search for new compounds by allowing the full use of rational drug design early in the screening process. Knowledge of target structure (or at least the active site) at the atomic level promotes the rapid development of rational drug design processes and speeds the search for new chemical entities with the proper structural characteristics for an optimal drug candidate. Ideally, the parasite target would not be homologous to human processes. This optimizes specificity and minimizes the chance of adverse drug effects. Finally, the target should be expressed and utilized at a stage of the parasite life cycle that is accessible to drug interdiction.

5.2 WRAIR's Genomics Approach to Target Identification.

The entire genome of *P. falciparum* has been sequenced. Figure 2 diagrams the broad approach that the Drug Discovery program at WRAIR has taken to leverage the genomic information in that sequence into productive new targets.

Parasite cultures are subjected to a variety of antimalarial compounds. Genes who's expression are modulated as a result of those exposures are identified. The set of genes that are up- or down-regulated in all treatments contains genes involved in nonspecific stress-related responses (red striped area in figure 2). Those sets of genes which are modulated in only one treatment condition are more likely to contain genes for proteins that are specifically involved in the response to that treatment.

The linkage of these specifically associated genes with known functional proteins may give an indication of what cellular processes are involved in the response to drugs. Thus, if treatment with drug C results in increased expression if genes involved in calcium regulation, careful examination of all components likely to be involved in that regulatory pathway a is warranted.

5.3 How are Expression Profiles Determined?

DNA specifically representing 7,256 parasite genes is spotted onto a micro chip the size of a standard microscope slide (figure 3).

FIGURE 2

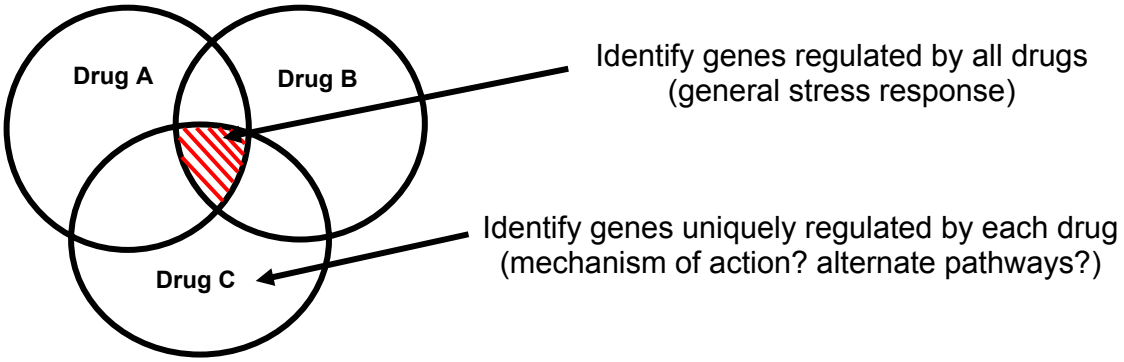

Fig. 2 Diagram of an approach to target identification.

The representative gene fragments of the oligo-nucleotide library are distributed to specific loci on the chip using a robotic microarray printer (figure 3 left panel). Each locus is then identified with a specific gene (figure 3 right panel).

FIGURE 3

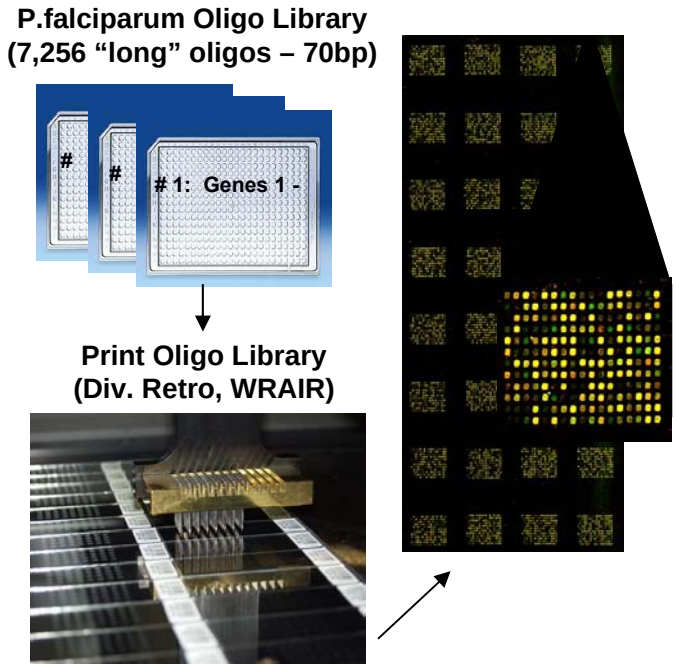

Fig. 3 Microarray of *P. falciparum* genome.

Messenger RNA is isolated from untreated parasites and labeled with a red dye. Messenger RNA is isolated from parasites treated with a drug that inhibits a parasite target and labeled with a green dye. Both RNA isolates are added to the array and the two isolates compete for binding to each spot representing individual genes (figure 4). If a gene is upregulated during drug treatment,

FIGURE 4

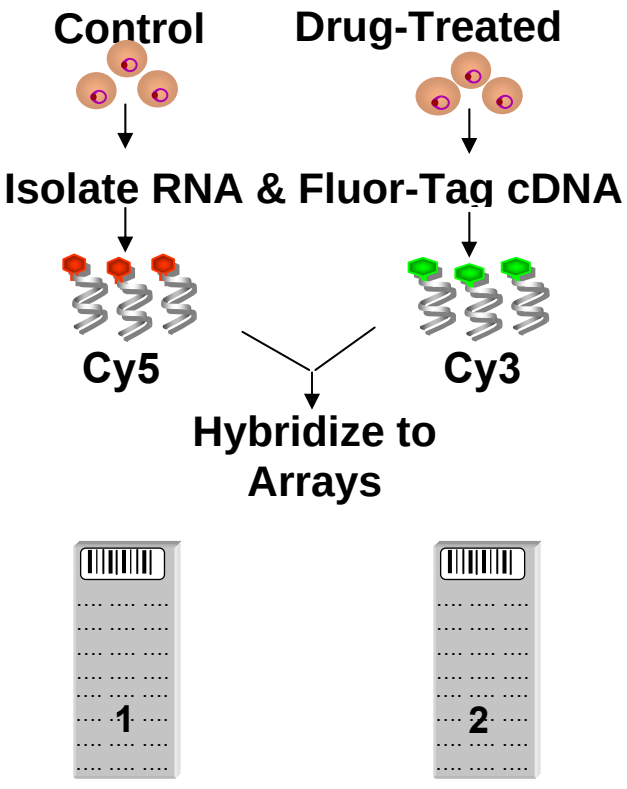

Fig. 4 Gene Expression Profiling

more of the green-tagged RNA will bind to the spot and it will be green. If the gene is turned off during drug treatment, more red-tagged RNA will be available to bind than green and the spot will be red. If there is no change in expression, there will be equal levels of red and green RNA bound and the array reader reports this as yellow.

This type of experiment is feasible only through the use of the microarray technology. The technology permits the simultaneous assessment of the expression activity of virtually all genes in the parasite. It is rapid, reproducible and amenable to alterations in experimental design. The technology also requires a tremendous computational capacity for data storage and analysis.

6. Rational Drug Design

6.1 The Pharmacophore

As drugs are tested in the target directed screens, their activities are related their structural characteristics. Given enough sets of structure/function correlations, sophisticated computer programs generate a mathematical model (pharmacophore) of the structural requirements for drug-target interaction. Figure 5 illustrates an example of a graphic representation of a pharmacophore. The green spheres represent areas in which hydrogen bond must occur for a molecule to exhibit efficacy in the *in vitro* parasite screen. Analogously, the blue spheres delineate the space in which aromatic hydrophobic interactions between the target and the drug must occur for high efficacy *in vitro*. Every time a new drug is tried the pharmacophore is improved. Figure 5 shows a pharmacophore developed using around 20 iterations of rational drug design. The pharmacophore can be used to search chemical libraries for compounds that fit it. Chemists may also modify available compounds to better fit the pharmacophore.

FIGURE 5

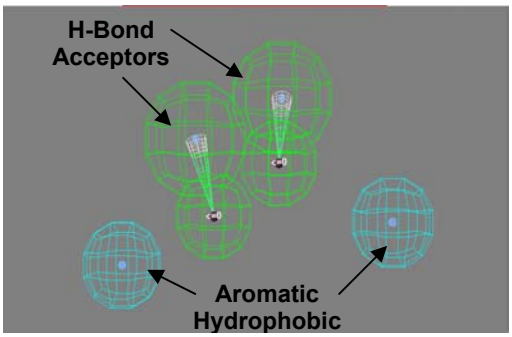

Fig. 5 Three dimensional pharmacophore

The new drug is tested and the process repeats itself. Rational drug design can be used in conjunction with any of the test systems (i.e. a pharmacophore for mammalian cell toxicity can be generated, facilitating drug modifications which might be less toxic). Therefore, the rational drug design process is used at multiple steps of the drug discovery process.

6.2 Utility of Rational Drug Design

Once a pharmacophore is established, chemists can use it to search chemical library data banks for compounds that fit it. Figure 6 illustrates the power of the rational drug design approach. In panel A, a member of a class of chemicals known to be efficacious in the *in vitro* parasite screen is shown to occupy all the required sites for efficacy. Panel B shows a compound from a drastically different class of compounds. Its close fit in the pharmacophore predicted that it would be efficacious in vitro. The chemical was subsequently tested and the efficacy was actually better than the original compound.

FIGURE 6

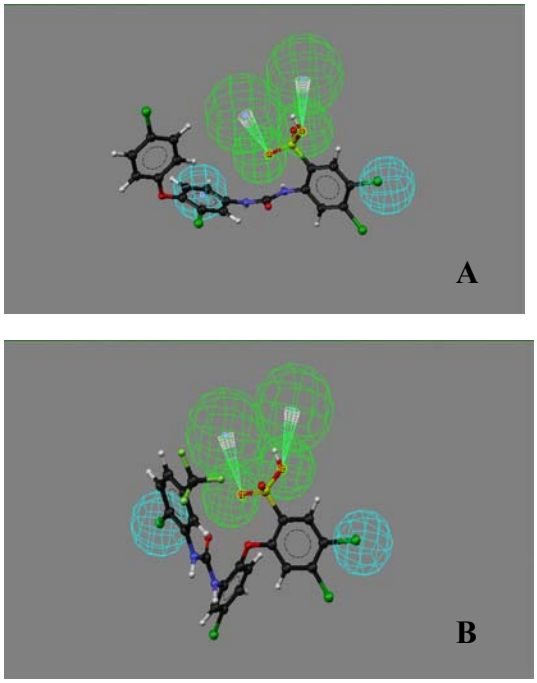

Fig. 6 Two chemically diverse compounds fit the pharmacophore and are potent antimalarial compounds

Another use for the pharmacophore is the elimination of compounds from the pool of possible test agents. Figure 7 shows two compounds superimposed on the same pharmacophore as shown in figure 6. Notice that

FIGURE 7

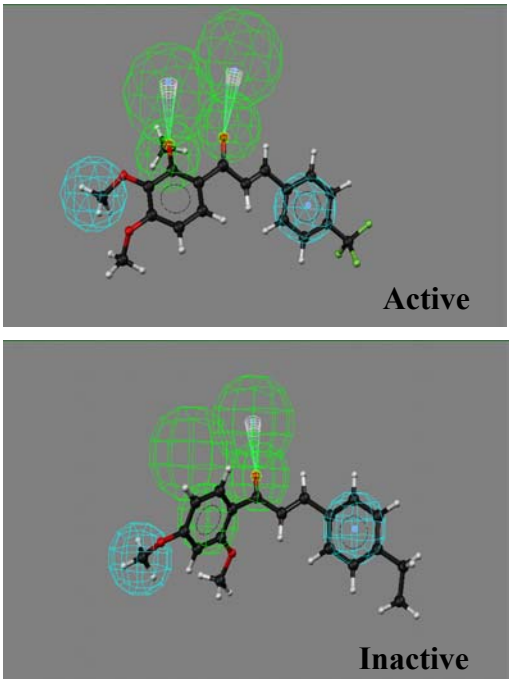

Fig. 7 Two structurally similar drugs which fit the pharmacophore differently.

The active drug fills all of the pharmacophore requirements while the inactive drug (which is very similar structurally to the active compound) does not fill one of the hydrogen bond requirements.

CONCLUSIONS

A description of the Antimalarial drug discovery program has been presented. The plan leverages the powerful technologies of molecular biology, genomics, computer modeling and high throughput screening into a target directed search for new compounds with a high probability of success in future deployment.

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